Production of Pyroglutamic Acid by Thermophilic Lactic Acid Bacteria in Hard-cooked Mini-Cheeses

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ABSTRACT

Pyroglutamic acid is present in high amounts (0.5g/100g) in many cheese varieties—and particularly in extensively ripened Italian cheeses such as Grana Padano and Parmigiano Reggiano.

An in vivo model system for cooked mini-cheese production and ripening acceleration was set up to demonstrate the ability of thermophilic lactic acid bacteria, used as a starter, to produce pyroglutamic acid (pGlu). In mini-cheeses stored at 38 and 30°C for up to 45 d, all starters tested produced different amounts of pGlu. In descending order of pGlu production, the bacteria analyzed were: *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, and *Lactobacillus delbrueckii* subsp. *lactis*.

Evidence for the presence of glutamine to pGlu cyclase activity in lactic acid bacteria was provided. Cell lysates obtained from cultures of *L. helveticus*, *L. delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* subsp. *lactis*, and *S. thermophilus* showed the ability to cyclize glutamine to pGlu, resulting in processing yields from 1.4 to 30.3%, depending on the subspecies. Formation of pGlu from free glutamine appeared to be similar to that observed using a glutamine-glutamine dipeptide substrate. Under the experimental conditions applied, pGlu aminopeptidase activity was only detected in *L. helveticus*. Thus, pGlu formation in long-ripened cooked cheese may depend on the activity of thermophilic lactic acid bacteria.

(Key words: pyroglutamic acid, lactic acid bacteria, cyclase activity)

Abbreviation key: LAB = Lactic Acid Bacteria, NCN = noncasein nitrogen, pGlu = Pyroglutamic Acid, PCP = pyrrolidone carboxyl peptidase, PYRase = L-pyroglutamil-peptide hydrolase.

INTRODUCTION

Pyroglutamic acid (pGlu) (5-oxo-proline), or pyrrolidone carboxylic acid, is found either as a free acid or at the N terminus of various proteins, and the localization of pGlu at the N terminus of various bioactive peptides is associated with their biological activity (Awadé et al., 1994), e.g. protecting proteins or peptides against degradation (Van Coillie et al., 1998). The pGlu has been shown to have pharmacological properties (Awadé et al., 1994), e.g. as an excitatory amino acid antagonist (Beani et al., 1990), brain stimulator (Mirzozian et al., 1994), and anxiolytic agent (Beni et al., 1988).

pGlu can also be released in cheese from the N terminus of proteins and peptides by the activity of pyrrolidone carboxyl peptidase (PCP) or L-pyroglutamil-peptide hydrolase (PYRase) (E.C. 3.4.11.8). PCP has been detected in many bacteria (Awadé et al., 1994; Williams et al., 1997; Laan et al., 1998; Williams and Banks, 1998). The enzymatic synthesis of pGlu by cyclotransferase from glutamic acid or glutamine as precursors has been described for *Pseudomonas cruciviae* (Akita et al., 1959) and *Streptococcus bovis* (Chen and Russell, 1989).

As a free acid in the enantiomeric form L, pGlu has been found in large quantities in Parmigiano-Reggiano and Grana Padano cheeses, two typical Italian hard-cooked cheeses produced using a natural whey starter composed mainly of thermophilic lactobacilli and ripened for a minimum of 12 and 9 months respectively (Mucchetti et al., 2000). After 2 to 3 mo of ripening, pGlu appeared and progressively increased for up to 24 mo of ripening and 600 mg/100 g of cheese (Panari, 1985; Mucchetti et al., 2000).

That thermophilic lactobacilli are involved in pGlu production is suggested by the low content of pGlu (67 mg/100 g) found in Bagos, a long-ripened Italian mountain cheese made without starter addition, and by the limited pGlu content (150 mg/100 g) detected in cheese made from starters containing *S. thermophilus* after 5 to 6 mo of ripening (Mucchetti et al., 2000). pGlu content in experimental Grana Padano cheeses made with raw or pasteurised milk was similar (Mucchetti et al., 2000).

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Table 1. pH, salt, and galactose content of mini-cheeses after brining within 24-h of cheese making

Starter	Code	NaCl	Galactose	pH
Commercial whey starter	SG	1.29 ± 0.028 ^a	0.00 ^a	5.26 ± 0.028 ^a
<i>L. helveticus</i>	LH	1.13 ± 0.057 ^b	0.06 ± 0.014 ^a	4.78 ± 0.014 ^b
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	LB	1.51 ± 0.071 ^c	0.95 ± 0.042 ^b	5.15 ± 0.042 ^c
<i>L. delbrueckii</i> subsp. <i>lactis</i>	LL	1.20 ± 0.028 ^{ab}	0.04 ± 0.006 ^a	4.89 ± 0.057 ^d
<i>S. thermophilus</i>	ST	1.18 ± 0.042 ^{ab}	0.85 ± 0.028 ^c	5.42 ± 0.014 ^e
<i>S. thermophilus</i> + <i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	ST+LB	1.26 ± 0.028 ^a	0.88 ± 0.028 ^c	5.23 ± 0.028 ^{ac}

^{a,b,c,d,e}Values in the same column without a common letter are significantly different ($P < 0.05$).

These observations suggest that pGlu formation depends mainly on the whey starter microflora rather than that of raw milk (Mucchetti et al., 2000).

Because of the localization in cytoplasm of those enzymes able to release pGlu from the N terminus of peptides (Awadé et al., 1994), it can be supposed that these enzymes may be active in the cheese, mainly if bacterial cells undergo lysis. Hence, to release pGlu in cheese, the starter and/or raw milk microflorae must either undergo autolysis or be lysed. In cheese, Gatti et al. (1999) showed the presence of aminopeptidase activities, which are characteristic of thermophilic lactobacilli from whey starters and may be considered as markers of bacterial autolysis.

The presence of aminopeptidase pGlu activity has been rarely shown in thermophilic lactobacilli and streptococci. Williams and Banks (1997) and Williams et al. (1998) detected aminopeptidase pGlu activity by cell lysates from two *L. helveticus* strains, but did not find it in *L. delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis*.

This work aims to show the ability of thermophilic lactic acid bacteria (**LAB**) to form pGlu both in vivo and in vitro.

MATERIALS AND METHODS

In order to verify the in vivo role played by thermophilic LAB in pGlu production in hard long ripened cheeses, a model was set up using mini-cheeses made with different starters of defined composition and ripened at high temperatures to accelerate the bacterial cell lysis, and consequently the formation of pGlu.

Starters Used for the Production of Mini-cheeses

For the cheeses coded LH, LB, and LL, three single-species LAB starters, obtained by respectively mixing 4 strains of *Lactobacillus helveticus* 3, 7, 22 and 23 (LH), 5 strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* 2, 10, 15, 17 and 19 (LB), and 5 strains of *Lactobacillus delbrueckii* subsp. *lactis* 14 to 18 (LL), from the ILC collection, were prepared (Table 1). A commercial *Streptococcus thermophilus* culture (IDC, Centro Speri-

mentale Latte, Zelo Buon Persico, Milano, Italy) as a direct-to-vat starter, and *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* cultures (IDC, Centro Sperimentale Latte) were used for mini-cheeses coded ST and ST+LB. For the production of the reference mini-cheese (SG), a common commercial whey starter for industrial Grana Padano cheese making, containing undefined thermophilic lactobacilli (SierGrana, Alce, Novara, Italy), was used.

Cheese Vat Milk Processing Step

After a series of preliminary trials (not described here) aimed at setting up a production technique, six cheesemaking trials were carried with the aforementioned starters.

330 kg of milk partially skimmed (2.4% fat) by spontaneous creaming was pasteurized directly in a coagulation vat using the following thermal cycle: the heating time from 15 to 70°C was 15 min, the holding time at 70°C was 1 min, and the cooling time from 70°C to the renneting temperature of 32°C was 22 min. After adding 6 g of lysozyme (Clerici, Cadorago-Como, Italy) and the starter, coagulation occurred within 12 to 14 min by adding 4 g calf rennet (Titre 1:125,000, Clerici, Cadorago-Como, Italy) per 100 kg of milk. Following the Grana cheese making process (Neviani and Carini, 1994), after fine curd breakage and cooking at 53°C, the cooked curd was extracted to make mini-cheeses.

Curd Extraction and Molding

Each mini cheese was made from a 4-kg mixture containing curd and whey which was taken from the bulk maintained under stirring, and placed directly into a baby Gouda mold. The molds were provided with a net for rapid whey drainage. A lid was put on the mold so that mini-curds could be turned over without coming into contact with the operator's hands. Twenty cheeses were obtained from each vat. The overall curd extraction time to obtain the mini-cheeses was 5 min, and the remaining curd yielded a whole cheese. The mini-curds in the molds were placed in a forced-air oven at 40°C for 18 h and then immersed in saturated brine at

40°C for 1 h. After removal from the brine, the mini-cheeses were kept in the oven for 60 to 90 min to dry the residual brine on the surface. The dried cheeses were then immersed in a fused paraffin bath (hardening interval 68 to 74°C) to make a hydrophobic barrier, thus decreasing loss of weight by evaporation during the subsequent ripening step. Each cheese weighed approximately 300 g.

Ripening

Cheeses were weighed prior to ripening, and those exceeding $\pm 5\%$ of the average weight were rejected. To accelerate bacterial cell lysis, and consequently the cheese ripening and pGlu formation, the mini-cheeses, subdivided into 6-unit batches for each incubation temperature, were ripened in air ovens at 15°, 30°, and 38°C for up to 45 d.

Cheese Composition Analyses

The total solids content of the mini-cheeses was determined according to IDF No 4A:1982 standard (IDF, 1982). The NaCl content of cheese was determined according to IDF No 17A: 1972 standard (IDF, 1972).

To measure the acceleration of cheese ripening due to storage temperature, the total nitrogen, noncasein nitrogen (NCN), and NPN of the reference cheeses (SG), were evaluated according to Gripon et al. (1975).

The pGlu, lactic acid, and galactose contents of the cheeses were determined by HPLC with the method described by Bouzas et al. (1991) for the simultaneous analysis of sugars and organic acids in cheese, using an Aminex HPX-87 column (Bio-Rad Laboratories, Richmond, CA) and ultraviolet and refractive index detectors in series.

Microbiological Characteristics

Thermophilic LAB counts were carried out in MRS agar (Biokar, Beauvais, France) with anaerobic incubation of the poured plates at 42°C for 48 h. Total coliform counts were carried out in Violet Red Brilliant Agar (Oxoid, Basingstoke, UK) with incubation at 30°C for 24 h. Eumycetic microflora count was carried out in Oxytetracycline Glucose Yeast Extract Agar (Oxoid), and incubated at 25°C for 5 d.

Evaluation of Cell Lysis by Determination of Aminopeptidase Activity

To verify the influence of storage temperature on the degree of starter bacterial cell lysis, the amount of lysis was evaluated by determining the aminopeptidase ac-

tivity with lysine β -naphthylamide as a substrate. The enzymatic activity was only determined in cheeses produced with single-species multiple-strain cultures after 12 and 45 d of storage at 12°, 30°, and 38°C and was ascertained by the previously described method (Gatti et al., 1999).

Detection of Cyclase and Aminopeptidase pGlu Activities

In order to verify the ability of LAB to produce pGlu in vitro, the cyclase and peptidase activities of single strains of thermophilic lactobacilli and *S. thermophilus*, chosen among those used in the formulation of the starters, have also been evaluated.

L. helveticus 3, *L. delbrueckii* subsp. *lactis* 18, *L. delbrueckii* subsp. *bulgaricus* 10 and *S. thermophilus* A, isolated from the commercial blend used as a starter, were cultured in 400-ml MRS and M17 (Oxoid, UK) broth at 42°C after incubation overnight. After centrifugation at $11,600 \times g$ at 4°C for 30 min, the cells were washed twice using 50 mM glycerophosphate buffer, pH 7.0. The pellet was resuspended in 10 ml of 10 mM Tris Ethanol Amine (TEA) buffer, pH 7.0, then subjected to treatment in a French Press apparatus (20 K Manual Fill cell pressure, Spectronic Instruments, Analytical Control SpA, Cinisello Balsamo, Milano, Italy) at 4°C and 128 MPa for 15 (first run) and 5 min (second run). The cell lysate was frozen at -20°C.

Cyclase Activity: Conditions for Reaction and Determination of pGlu

Cell lysate (0.2 ml) was added to 1.8 ml of either 7.85 mg/ml of glutamine or 7.7 mg/ml of glutamine-glutamine dipeptide (Fluka Chemie AG, Sigma-Aldrich, Milano, Italy) dissolved in 10 mM TEA buffer, pH 8.6. The enzyme-substrate mixtures were then incubated at 42°C for 24 and 48 h. After ultrafiltration by centrifugation at 3000 g at 25°C for 95 min in Centriplus 3 MW microconcentrators with a 3000-dalton cutoff (Amicon, Passirana di Rho, Milan, Italy), the pGlu content of the filtrate was determined by HPLC, as described above.

Aminopeptidase pGlu Activity

Using the same cell lysates as those used to determine cyclase activity, the PYRase activity was determined by both spectrophotometry, using pGlu β -naphthylamide as a substrate as previously described (Gatti et al., 1999), and by high resolution chromatography. Cell lysate (0.1 ml) was added to 0.9 ml of solution containing 0.22 mg of pGlu-alanine peptide (Fluka) dis-

solved in 10 mM TEA buffer, pH 7.0. The enzyme-substrate mixture was incubated at 42°C for 24 h. After ultrafiltration by centrifugation at 3000 g at 25°C for 95 min in a Centriplus 3 MW microconcentrator with a 3000-dalton cutoff (Amicon), the pGlu content of the filtrate was determined by HPLC as described above.

Statistical analysis

Data from chemical cheese composition were evaluated for their significance ($P < 0.05$) with one-way ANOVA, by the software "Analyse-It+General Statistics vsn. 1.62" for Microsoft Excel (Analyse-It Company, UK).

RESULTS AND DISCUSSION

Mini-cheese Production

Except for the commercial SierGrana whey culture, all the mini-cheeses were made using starters containing defined thermophilic LAB. In 24-h cheeses LB and ST+LB, made using the starter containing *L. delbrueckii* subsp. *bulgaricus* and/or *S. thermophilus*, 0.95 and 0.85% nonfermented galactose was still present, respectively. Within 18 h of incubation at 40°C, the pH of mini-cheeses decreased to a range of 4.78 to 5.42, depending on the starter used (Table 1). The decrease in curd pH obtained with *L. helveticus* and *L. delbrueckii* subsp. *lactis* was higher than that occurring in Grana Padano. Despite the unusual temperature conditions (Giraffa et al., 1998), the decrease in pH demonstrated that the starter microflora was also viable under the above growth conditions, as confirmed by counts (see section). Processes for extraction, curd molding and ripening at different temperatures were developed, allowing us to obtain six series of mini-cheeses with an average total solids content of 58.59 ± 0.61 . Only the cheese LL, produced with *L. delbrueckii* subsp. *lactis* as a starter, showed a significantly different total solid content ($P < 0.05$) (Table 2).

Brining at 40°C (to accelerate the salt penetration without breaking the successive thermal cycle of ripening for the cheeses incubated at 38°C) allowed us to obtain cheeses having an average NaCl content of $1.26\% \pm 0.15$, which is similar to the amount usually found in Grana Padano and Parmigiano-Reggiano cheeses. The brining increased the differences in total solids contents among the six lots of mini-cheeses (Table 2). Ripening at high temperatures forced us to use cheese rind paraffin to reduce moisture loss by evaporation, especially for cheeses ripened at 30 and 38°C. However, the variation in evaporation for the six lots of mini-cheeses resulted in different amounts of total solids among the cheeses ripened at the three temperatures (Table 2).

Means for the total solids content of cheeses within 45 d of ripening ranged from the initial value of $58.59\% \pm 0.61$ to 60.87 ± 0.97 , 64.49 ± 2.35 and $66.05\% \pm 1.89$ for the cheeses ripened 45 d at 15, 30 and 38°C, respectively. These differences in the total solids content of mini-cheeses during ripening fit well with those of traditional ripening of Grana Padano and Parmigiano-Reggiano cheeses which have total solids values around 58 to 60% prior to salting and around 67 to 70% after 12 mo.

Microbiological Characteristics of Mini-cheeses

It was possible to distinguish between the development of the lactic microflora added with the starter during the first 24 h of cheesemaking and the development at the end of the ripening period at the three different temperatures (Table 3). Cheeses LH and LB, made from *L. helveticus* and *L. delbrueckii* subsp. *bulgaricus*, showed decreased values for cfu/g after 24 h, compared with those found in cooked curds. Conversely, the other mini-cheeses showed a progressive increase in the lactic microflora from the starter. The ripening temperature sharply decreased the viable microbial population in cheeses ripened at 38°C, except for those made from commercial starters (containing undefined thermophilic lactobacilli, or a mixture of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*), where cell death was definitely lower. The presence of nonfermented galactose in the 24-h cheeses ST, LB and ST+LB made with starters containing galactose-negative *L. delbrueckii* subsp. *bulgaricus* or *S. thermophilus* strains (Kandler and Weiss, 1986), together with the absence of coliform and eumycetic microflora, demonstrated that the prevailing microflora of mini-cheeses was supplied by starters.

Evaluation of Accelerated Ripening in Reference Cheeses

Storage of reference cheeses (SG) at 30° and 38°C resulted in a considerable increase in the NCN/NT (21.5 vs. 11.3%) and NPN/NCN (100 vs. 91.4%) ripening indices compared with cheeses ripened at 15°C (Table 4). The NCN/NT index for the cheeses stored at 38°C after 45 d increased to 23%. This corresponds to the value for Parmigiano-Reggiano cheese after 8 mo (Panari et al., 1988). In addition, an NPN/NCN ratio close to one suggests that peptides deriving from the action of proteases on casein are quantitatively hydrolyzed to oligopeptides and amino acids by peptidil-peptidases and/or aminopeptidases. Acceleration in ripening was induced by a faster release of intracellular enzymes to the cheese paste.

Table 2. Influence of brining and ripening temperatures on the increase of total solids in mini-cheeses.

Days ripened	SG	LH	LB	LL	ST	ST+LB
1d before brining	59.38 ± 0.25 ^a	58.11 ± 0.33 ^a	58.27 ± 0.33 ^a	57.93 ± 0.20 ^b	58.65 ± 0.25 ^a	59.20 ± 0.28 ^a
1d after brining	60.96 ± 0.34 ^a	60.18 ± 0.34 ^{ab}	60.99 ± 0.26 ^a	60.92 ± 0.34 ^a	61.88 ± 0.35 ^c	61.70 ± 0.35 ^c
Ripening at 38°C						
12	62.27 ± 0.26 ^a	61.15 ± 0.22 ^b	63.41 ± 0.36 ^{ac}	61.77 ± 0.26 ^{ab}	64.66 ± 0.14 ^d	64.60 ± 0.23 ^d
20	64.55 ± 0.37 ^a	62.86 ± 0.36 ^b	65.14 ± 0.28 ^{ac}	62.56 ± 0.22 ^b	63.76 ± 0.16 ^{ab}	64.67 ± 0.37 ^a
30	66.65 ± 0.14 ^a	64.45 ± 0.23 ^b	65.78 ± 0.23 ^c	65.38 ± 0.14 ^c	63.95 ± 0.14 ^b	66.69 ± 0.14 ^a
45	67.51 ± 0.17 ^a	64.23 ± 0.27 ^b	65.38 ± 0.23 ^c	64.69 ± 0.37 ^{bc}	65.14 ± 0.37 ^{bc}	69.33 ± 0.25 ^d
Ripening at 30°C						
12	62.64 ± 0.13 ^a	60.65 ± 0.21 ^b	60.67 ± 0.15 ^{bc}	61.05 ± 0.22 ^{bc}	62.14 ± 0.35 ^{ac}	61.62 ± 0.26 ^{bc}
20	63.67 ± 0.16 ^a	60.09 ± 0.34 ^b	61.88 ± 0.35 ^c	61.66 ± 0.16 ^c	62.47 ± 0.27 ^c	61.28 ± 0.16 ^c
30	66.51 ± 0.38 ^a	60.88 ± 0.13 ^b	62.09 ± 0.16 ^c	62.61 ± 0.22 ^c	64.44 ± 0.16 ^d	62.65 ± 0.35 ^c
45	66.50 ± 0.17 ^a	62.14 ± 0.26 ^b	65.35 ± 0.23 ^c	61.56 ± 0.04 ^b	67.71 ± 0.38 ^d	63.66 ± 0.16 ^e
Ripening at 15°C						
12	62.17 ± 0.35 ^a	59.52 ± 0.15 ^b	60.68 ± 0.34 ^{ac}	60.73 ± 0.13 ^c	61.17 ± 0.35 ^{ac}	61.64 ± 0.35 ^{ac}
20	62.13 ± 0.13 ^a	59.99 ± 0.34 ^b	60.68 ± 0.15 ^b	61.33 ± 0.16 ^c	61.14 ± 0.26 ^{bc}	60.28 ± 0.13 ^b
30	63.09 ± 0.27 ^a	59.91 ± 0.21 ^b	61.21 ± 0.22 ^c	61.79 ± 0.26 ^c	61.88 ± 0.35 ^c	60.84 ± 0.34 ^{bc}
45	63.11 ± 0.22 ^a	61.08 ± 0.13 ^b	60.33 ± 0.26 ^b	60.53 ± 0.34 ^b	61.97 ± 0.11 ^c	61.59 ± 0.26 ^c

^{a,b,c,d,e}Values in the same row without a common letter are significantly different ($P < 0.05$).

Evaluation of Cell Lysis

Cheeses ripened at 30°C always showed a higher residual aminopeptidase activity on lysine β -naphthylamide than those ripened at 15°C. Cheeses stored at 38°C showed the higher activity after 12 d, while the activity lessened after 45 d. Extending the storage to 45 d at 38°C likely reduced the detectable residual enzymatic activity, which was generally lower than that determined in cheeses stored at 30 and 15°C (Table 5). We suggest that this reduction cannot be due to a reduced bacterial lysis, but to a reduction in stability of some enzymes able to hydrolyse lysine β -naphthylamide. Cheese ripening at high temperatures accelerated cell lysis, as previously observed in vitro (Long et al., 1999) and in hard semicooked cheeses (Gatti et al., 2000).

pGlu Production

Ripening cheese at 38°C and 30°C accelerated pGlu production. In all the mini-cheeses, pGlu was detectable

after 12 d incubation at 38°C (Table 6). Ripening at 30°C also accelerated pGlu production in all cheeses except for cheese ST. ST was made using the starter containing *S. thermophilus*, and pGlu did not appear until after 20 d. In the mini-cheeses, *L. helveticus* and, to a smaller extent, *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* formed pGlu the easiest. pGlu production was comparable to that detected in the reference cheese (SG) made using a commercial whey starter containing undefined thermophilic lactobacilli. *L. delbrueckii* subsp. *lactis* showed much less of an ability to form pGlu. The combination of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* did not synergistically enhance pGlu formation. In the cheeses ripened at 15°C, pGlu production was absent or negligible until d 30, and after 45 d pGlu content was still lower than 40 mg/100 g. Using the pGlu content as a ripening marker (Panari, 1985; Mucchetti et al., 2000), mini-cheese ripening at 38°C for 45 d corresponded to 10 to 11 mo of ripening under standard conditions. The importance of the thermophilic lactic microflorae for pGlu production

Table 3. Thermophilic lactic acid bacteria growth on MRS agar during the first 24 h of mini-cheese production and after 45 d of ripening at temperature of 15°, 30°, and 38°C.

Code	Milk + starter	Cooked curd	24h	Cheese aged 45d		
				15°C	30°C	38°C
LH	9.90E + 06	6.48E + 07	2.43E + 06	1.73E + 06	5.29E + 06	2.47E + 05
LL	3.70E + 06	2.08E + 07	1.77E + 08	4.47E + 06	1.00E + 04	1.00E + 04
LB	3.15E + 06	3.96E + 07	4.00E + 06	1.85E + 06	1.38E + 06	1.00E + 04
ST	5.44E + 06	1.40E + 08	1.00E + 08	5.34E + 06	3.83E + 06	3.87E + 04
ST+LB	3.10E + 05	6.06E + 06	1.49E + 08	3.36E + 06	1.34E + 07	1.68E + 06
SG	9.67E + 05	2.94E + 06	2.22E + 07	1.69E + 06	2.39E + 06	2.96E + 06

Table 4. Total nitrogen (TN), noncasein nitrogen (NCN), nonprotein nitrogen (NPN) and ripening indices of the reference mini-cheeses (SG) after 30 d of ripening at temperature of 15°, 30°, and 38°C

Ripening temperature	Total Nitrogen	NCN	NPN	NCN/TN %	NPN/NCN %
°C	g/100g	g/100g	G/100g		
38	5.32	1.15	1.15	21.5	100
30	5.25	0.95	0.94	18.0	98.9
15	5.12	0.58	0.53	11.3	91.4

in Grana Padano and Parmigiano-Reggiano cheeses was confirmed by the fact that the average pGlu production in the mini-cheeses was comparable to that obtained in reference mini-cheeses made with commercial whey starters containing undefined thermophilic lactobacilli and in commercial mature Grana Padano cheese. Differences in activities between the genera and/or species may help understand the differences observed in pGlu content between Grana Padano cheeses having the same age and produced using different whey starters (data not shown).

Detection of Cyclase Activity in Thermophilic LAB

The late appearance of pGlu in Grana Padano and Parmigiano-Reggiano cheeses after at least 2 mo of ripening may depend on intracellular localization of the enzymatic systems involved in pGlu formation and a lack or limited presence of its precursors. Intracellular localization of common pyrase and cyclase enzymes is reported in the literature (Awadé et al., 1994), whereas partial, incomplete information is available on pyrases of LAB (Williams and Banks, 1997; Williams et al., 1998; Laan et al., 1998).

This work confirmed the association of glutamine to pGlu cyclase activity in LAB. We verified whether cell lysates produced pGlu from either free glutamine or glutamine-glutamine dipeptide in cultures of the strains *L. helveticus* 3, *L. delbrueckii* subsp. *bulgaricus* 10, *L. delbrueckii* subsp. *lactis* 18 and *S. thermophilus* A (Table 7). Cyclization of free glutamine in vitro appeared to be similar to that observed using glutamine-glutamine peptide as a substrate. Hence, under the

Table 5. Residual aminopeptidase activity on lysine β -naphthylamide detected in cheeses produced with single species-multiple strain starter after 12 and 45 d of ripening at temperature of 15°, 30°, and 38°C

Starter	12 d			45 d		
	15°C	30°C	38°C	15°C	30°C	38°C
LH	1.419	1.485	1.6	1.038	1.577	1.37
LL	0.112	0.161	0.209	0.136	0.31	0.30
LB	0.334	0.517	0.544	0.412	0.615	0.33
ST	0.112	0.191	0.02	0.122	0.231	0.03

conditions applied, conversion of glutamine to pGlu varied between 1.4 and 30.3%. *L. helveticus* 3, *L. delbrueckii* subsp. *bulgaricus* 10, and *S. thermophilus* A all showed a similar ability to form pGlu, whereas *L. delbrueckii* subsp. *lactis* 18 had a much lesser ability.

It is still not possible, based on these data, to establish if pGlu in cheese originated from free glutamine (and/or glutamic acid) and/or from peptides having N-terminal glutamine. pGlu precursors in cheese have not yet been clearly identified. During Grana Padano cheese ripening (Borghi, 1999), glutamic acid accumulates in the free amino acid fraction up to a relative percentage of about 30%, which is far beyond the relative percentage of glutamic acid in casein [about 10% (Alais, 1984)]. Conversely, a decrease in the glutamine content to values lower than 1% was observed in the same free fraction. Identification of peptides in Parmigiano-Reggiano cheese (Ferranti et al., 1997) showed a considerable amount of peptides, which had N-terminal glutamic acid and/or glutamine residue removable by aminopeptidase action.

Aminopeptidase pGlu Activity

Under experimental conditions, no aminopeptidase activity was detected using pGlu β -naphthylamide as a substrate. *L. helveticus* 3 was the only lysate that

Table 6. Kinetics of pGlu formation in cheeses produced with different starters and ripened at 15° (usual ripening temperature), 30°, and 38°C. Data expressed as mg pGlu/100 g total solids.

Days	SG	LH	LB	LL	ST	ST+LB
Ripening at 38°C						
12	325.5	223.3	223.9	74.8	102.5	151.1
20	403.0	392.9	336.7	123.7	287.6	166.0
30	515.6	531.7	441.5	216	349.2	281.0
45	593.0	674.4	525	221.9	510.7	516.1
Ripening at 30°C						
12	76.5	75.5	98.4	15.4	0.1	29.4
20	186.2	187.2	157.9	88.2	73.8	64.7
30	364.8	297.6	275.9	180.2	190.7	90.3
45	409.0	542.3	404.7	204.9	397.3	301.4
Ripening at 15°C						
12	0.0	0.1	0.1	0.1	0.1	0.1
20	5.0	0.1	0.1	0.1	0.1	0.1
30	20.2	0.1	6.3	0.1	0.1	0.1
45	37.2	6.5	23.8	0.1	0.1	0.1

Table 7. Glutamine and glutamine-glutamine to pGlu cyclase activity detected in four strains of thermophilic lactic acid bacteria after incubation of bacterial lysate at 42°C for 24, 30, and 48 h. Data expressed as mg pGlu/ml and percentage of conversion of glutamine and glutamine-glutamine to pGlu.

Strain	reaction time (h)	Gln		gln-gln	
		mg pGlu/ml	(pglu/gln)%	mg pGlu/ml	(pGlu/gln-gln)%
LH3	24	0.31	11.17	0.17	5.98
	30	0.75	27.02	0.68	23.94
	48	0.85	30.27	0.74	26.05
LL18	24	0.06	2.16	0.04	1.40
	30	0.25	9.00	0.20	7.04
	48	0.18	6.48	0.14	4.92
LB10	24	0.42	15.14	0.16	5.63
	30	0.53	19.10	0.57	20.07
	48	0.71	25.59	0.61	21.47
ST A	24	0.39	14.41	0.38	13.38
	30	0.47	16.58	0.49	16.90
	48	0.77	27.39	0.75	26.06

hydrolyzed pGlu from pGlu-alanine peptide. This result was not obtained with *L. delbrueckii* subsp. *bulgaricus* 10, *L. delbrueckii* subsp. *lactis* 18 or *S. thermophilus* A. Williams and Banks (1997) detected aminopeptidase activity on pyroglutamyl p-nitro anilide substrate by cell lysates from cultures of lactobacilli (which also included two *L. helveticus* strains) isolated from Cheddar cheese nonstarter microflora. Using cell lysates, Williams et al. (1998) confirmed pyrased activity in *L. helveticus* and in many mesophilic lactobacilli species, whereas they did not detect this activity in *L. delbrueckii* subsp. *bulgaricus* or *L. delbrueckii* subsp. *lactis*. Laan et al., 1998 also detected pyrased activity in *Lactobacillus casei*, *Lactobacillus plantarum*, and *Lactococcus lactis*, thus confirming previous observations by Exterkate and Stadhouders (1971).

CONCLUSIONS

pGlu is a cyclic amino acid, and its content is high in long-ripened cheeses made with starters containing thermophilic LAB (Mucchetti et al., 2000). Despite many studies on the pharmacological properties of pGlu, no data are available on the nutritional and/or pharmacological relevance of the amount of pGlu ingested with hard-cooked cheeses or other foods.

In a previous paper (Mucchetti et al., 2000), the presence of pGlu in different varieties of cheese was evaluated, and some hypotheses as to its formation were made. The present paper provides additional information on the role of thermophilic LAB in producing pGlu. The late appearance of pGlu in hard long ripened cheeses made it necessary to develop a system to accelerate cooked mini-cheese production and ripening in order to demonstrate in vivo pGlu production by the

starter microflora. The strains used to make starters showed the ability to produce pGlu in mini-cheeses according to the following order: *L. helveticus*, *L. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*, and *L. delbrueckii* subsp. *lactis*.

The association of glutamine with pGlu cyclase activity was demonstrated in vitro in the four thermophilic lactic species, and aminopeptidase activity was only detected in *L. helveticus*. It can thus be assumed that pGlu formation in long-ripened cooked cheese may depend on thermophilic LAB.

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